THE EFFECT OF SALT PROPORTIONS AND CONCENTRATION ON THE GROWTH OF ASPERGILLUS NIGER[1]

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INTRODUCTION

A great deal of work has been done in recent years on the various problems of plant nutrition, and especially on the physiological balance of nutrient solutions and on the salt requirements of plants. Although these questions have received some attention ever since the introduction of water cultures by Sachs and Knop, they had never been carried to such logical completeness as was done by Tottingham (9) in his work on the study of the effect upon plant growth of varying the total concentration and salt proportions in Knop's solution. In 1915 Shive (6), using Tottingham's systematic methods, made similar exhaustive studies of a nutrient solution containing only three salts and a trace of iron. This three-salt solution, when properly balanced, gave a better yield than Tottingham's best four-salt solution and has the further advantage of being the simplest satisfactory water culture that had been used up to that time.

Since the introduction of Shive's three-salt solution, which, on account of its relative simplicity, is especially suitable for studies in plant nutrition, it has been used extensively by Shive (6), McCall (4), Hibbard (3), and others for various physiological studies with green plants.

Fundamental problems of nutrition have also been studied extensively in certain fungi and especially in *Aspergillus niger*. The greater number of these studies have dealt with carbon or nitrogen assimilation and with the toxic or stimulative action of various substances. The rôle played by total salt concentration and by the salt proportions of the nutrient medium has apparently received very little attention. There has been no systematic study made upon any fungus which corresponds to the nutrition studies made with green plants by Tottingham, Shive, and others. The results of these authors have proven of such fundamental importance in nutrition studies in higher plants that it was thought advisable to test the adaptability of their methods in similar studies upon fungi.

In order to make this test, a series of experiments were planned to study the effect upon *Aspergillus niger* of varying the total salt concentration and salt proportions in a simple nutrient solution.

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The methods used by Shive (6) in his studies on the physiological balance of nutrient solutions for higher plants were used as a basis for this work. A number of radical modifications in these methods, however, were necessary on account of the physiological differences between the green plants and fungi. The most essential modification was in the composition of the nutrient solution itself. In Shive's solution three mineral salts and a trace of iron constitute the nutrient material, and for green plants these salts contain all the necessary elements for growth. For fungi, however, a source of energy must be supplied in addition to the mineral salts, and in this work it was supplied in the form of cane sugar. A second necessary modification was in the treatment of the solution. In water-culture work with higher plants it is neither essential nor practicable always to use sterile solutions. With the fungi, on the other hand, it is essential that the medium be sterilized thoroughly before inoculating with the desired organism. The sterilization process as used for these experiments, however, probably caused very little alteration of the medium and may be overlooked as a factor influencing the medium itself.

This work was outlined primarily to see whether the methods which Shive and others have used with such marked success in nutrition studies with green plants, would prove equally useful for similar studies with fungi.

This work was carried out under the direction of Dr. J. W. Shive in the Laboratory of Plant Physiology at the New Jersey Agricultural Experiment Station.

Experimental Methods

The experiments herein discussed consist of two groups of cultures. Group 1, comprising series 1, 2, and 3, contains $Ca(NO_3)_2$ as the source of nitrogen and will be referred to as the $Ca(NO_3)_2$ group. Group 2, comprising series 4 and 5, contains $NaNO_3$ as the source of nitrogen and will be designated the $NaNO_3$ group.

Aspergillus niger was grown in 250 cc. Jena glass Erlenmeyer flasks on 50 cc. of a liquid medium containing three nutrient salts, cane sugar, and a trace of iron. Each culture throughout five series contained the same amount of iron and sugar, the iron being present in amounts equivalent to 0.01 gram of ferrous sulphate per liter, and the sugar in amounts equivalent to 38.97 grams per liter of nutrient solution.

In series 1, the nutrient salts, KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$, are present in quantities sufficient to give a total calculated osmotic concentration value of 0.5 atmospheres. The series consists of 36 cultures, each differing from all the other cultures of the series in the proportions of the three nutrient salts. The 36 cultures represent all the possible proportions or combinations obtainable by varying the partial concentration of each of the salts by increments of one tenth of the total concentration. A full account of this method of studying the effects of salt proportions is given by Shive (6) and need not be further discussed here.

Series 2 and 3 differ from series 1 only in total salt concentration, the total calculated osmotic concentration values in these series being 2.1 and 4.2 atmospheres respectively. Series 4 and 5 have the same total concentration values as series 2 and 3 respectively, but in the former $NaNO_3$ is used instead of $Ca(NO_3)_2$. For purposes of comparison the standard nutrient solution proposed by Thom (8) was here used as a check in each series (except series 4), always with the same total osmotic salt concentration as that employed for the series in which it occurred. In Tables 1 and 2 are given the actual

TABLE 1. *Partial volume-molecular concentration of the salts employed in the culture solutions of the series in group 1*

Culture No.	Series 1 (0.5 Atm.)			Series 2 (2.1 Atm.)			Series 3 (4.2 Atm.)		
	KH_2PO_4	$Ca(NO_3)_2$	$MgSO_4$	KH_2PO_4	$Ca(NO_3)_2$	$MgSO_4$	KH_2PO_4	$Ca(NO_3)_2$	$MgSO_4$
R1C1	.00111	.00070	.00941	.00444	.00302	.04157	.00888	.00625	.08648
R1C2	.00111	.00141	.00824	.00444	.00604	.03637	.00888	.01250	.07567
R1C3	.00111	.00211	.00706	.00444	.00906	.03118	.00888	.01875	.06486
R1C4	.00111	.00282	.00588	.00444	.01208	.02598	.00888	.02500	.05405
R1C5	.00111	.00352	.00471	.00444	.01510	.02078	.00888	.03125	.04234
R1C6	.00111	.00423	.00353	.00444	.01811	.01559	.00888	.03749	.03243
R1C7	.00111	.00493	.00235	.00444	.02113	.01039	.00888	.04374	.02162
R1C8	.00111	.00563	.00118	.00444	.02415	.00520	.00888	.04999	.01081
R2C1	.00222	.00070	.00824	.00888	.00302	.03637	.01776	.00625	.07567
R2C2	.00222	.00141	.00706	.00888	.00604	.03118	.01776	.01250	.06486
R2C3	.00222	.00211	.00588	.00888	.00906	.02598	.01776	.01875	.05405
R2C4	.00222	.00282	.00471	.00888	.01208	.02078	.01776	.02500	.04324
R2C5	.00222	.00352	.00353	.00888	.01510	.01559	.01776	.03125	.03243
R2C6	.00222	.00423	.00235	.00888	.01811	.01039	.01776	.03749	.02162
R2C7	.00222	.00493	.00118	.00888	.02113	.00520	.01776	.04374	.01081
R3C1	.00333	.00070	.00706	.01332	.00302	.03118	.02664	.00625	.06486
R3C2	.00333	.00141	.00588	.01332	.00604	.02598	.02664	.01250	.05405
R3C3	.00333	.00211	.00471	.01332	.00906	.02078	.02664	.01875	.04324
R3C4	.00333	.00282	.00353	.01332	.01208	.01559	.02664	.02500	.03243
R3C5	.00333	.00352	.00235	.01332	.01510	.01039	.02664	.03125	.02162
R3C6	.00333	.00423	.00118	.01332	.01811	.00520	.02664	.03749	.01081
R4C1	.00444	.00070	.00588	.01776	.00302	.02598	.03552	.00625	.05405
R4C2	.00444	.00141	.00471	.01776	.00604	.02078	.03552	.01250	.04324
R4C3	.00444	.00211	.00353	.01776	.00906	.01559	.03552	.01875	.03243
R4C4	.00444	.00282	.00235	.01776	.01208	.01039	.03552	.02500	.02162
R4C5	.00444	.00352	.00118	.01776	.01510	.00520	.03552	.03125	.01081
R5C1	.00555	.00070	.00471	.02220	.00302	.02078	.04440	.00625	.04324
R5C2	.00555	.00141	.00353	.02220	.00604	.01559	.04440	.01250	.03243
R5C3	.00555	.00211	.00235	.02220	.00906	.01039	.04440	.01875	.02162
R5C4	.00555	.00282	.00118	.02220	.01208	.00520	.04440	.02500	.01081
R6C1	.00666	.00070	.00353	.02664	.00302	.01559	.05328	.00625	.03243
R6C2	.00666	.00141	.00235	.02664	.00604	.01039	.05328	.01250	.02162
R6C3	.00666	.00211	.00118	.02664	.00906	.00520	.05328	.01875	.01081
R7C1	.00777	.00070	.00235	.03108	.00302	.01039	.06216	.00625	.02162
R7C2	.00777	.00141	.00118	.03108	.00604	.00520	.06216	.01250	.01081
R8C1	.00888	.00070	.00118	.03552	.00302	.00520	.07104	.00625	.01081

partial volume-molecular concentrations of each of the solutions of the five series used in this study. The culture numbers refer to the positions which the cultures occupy on the triangular diagram[2] graphically representing the series with respect to the osmotic proportions of the three salts employed.

[2] For a description of this triangular diagrammatic scheme see Shive (6), McCall (4), Hibbard (3).

TABLE 2. *Partial volume-molecular concentration of the salts employed in the cultures of the series in group 2*

Culture No.	Series 4 (2.1 Atm.)			Series 5 (4.2 Atm.)		
	KH_2PO_4	$NaNO_3$	$MgSO_4$	KH_2PO_4	$NaNO_3$	$MgSO_4$
R1C1	.00444	.00414	.04157	.00888	.00860	.08648
R1C2	.00444	.00829	.03637	.00888	.01721	.07567
R1C3	.00444	.01243	.03118	.00888	.02581	.06486
R1C4	.00444	.01658	.02598	.00888	.03442	.05405
R1C5	.00444	.02072	.02078	.00888	.04302	.04324
R1C6	.00444	.02487	.01559	.00888	.05162	.03243
R1C7	.00444	.02901	.01039	.00888	.06023	.02162
R1C8	.00444	.03316	.00502	.00888	.06883	.01081
R2C1	.00888	.00414	.03637	.01776	.00860	.07567
R2C2	.00888	.00829	.03118	.01776	.01721	.06486
R2C3	.00888	.01243	.02598	.01776	.02581	.05405
R2C4	.00888	.01658	.02078	.01776	.03442	.04324
R2C5	.00888	.02072	.01559	.01776	.04302	.03243
R2C6	.00888	.02487	.01039	.01776	.05162	.02162
R2C7	.00888	.02901	.00502	.01776	.06023	.01081
R3C1	.01332	.00414	.03118	.02664	.00860	.06486
R3C2	.01332	.00829	.02598	.02664	.01721	.05405
R3C3	.01332	.01243	.02078	.02664	.02581	.04324
R3C4	.01332	.01658	.01559	.02664	.03442	.03243
R3C5	.01332	.02072	.01039	.02664	.04302	.02162
R3C6	.01332	.02487	.00502	.02664	.05162	.01081
R4C1	.01776	.00414	.02598	.03552	.00860	.05405
R4C2	.01776	.00829	.02078	.03552	.01721	.04324
R4C3	.01776	.01243	.01559	.03552	.02581	.03243
R4C4	.01776	.01658	.01039	.03552	.03442	.02162
R4C5	.01776	.02072	.00502	.03552	.04302	.01081
R5C1	.02220	.00414	.02078	.04440	.00860	.04324
R5C2	.02220	.00829	.01559	.04440	.01721	.03243
R5C3	.02220	.01243	.01039	.04440	.02581	.02162
R5C4	.02220	.01658	.00502	.04440	.03442	.01081
R6C1	.02664	.00414	.01559	.05328	.00860	.03243
R6C2	.02664	.00829	.01039	.05328	.01721	.02162
R6C3	.02664	.01243	.00502	.05328	.02581	.01081
R7C1	.03108	.00414	.01039	.06216	.00860	.02162
R7C2	.03108	.00829	.00502	.06216	.01721	.01081
R8C1	.03552	.00414	.00502	.07104	.00860	.01081

For each series a stock solution of each of the salts was made up with distilled water to such a concentration that 1 cc. of the stock in 50 cc. of the culture solution produced in any culture one tenth of its total required salt concentration. Thus, the culture in which the three salts KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$ produced, respectively, 1/10, 6/10, and 3/10 of the total concentration, received of these stocks 1 cc., 6 cc., and 3 cc., in the order given. Each culture, therefore, received a total of 10 cc. of stock solutions, leaving 40 cc. to be supplied by other means. The sugar stock was made up to 5/4 the concentration desired in the finished culture; thus, 40 cc. of this stock contained the proper amount of sugar for the 50 cc. culture. Ferrous sulphate equivalent to 0.01 gram $FeSO_4$ per liter of finished nutrient was added directly to the sugar stock.

The stock solutions were weighed and sterilized separately at 100° C. for one hour on three consecutive days. The loss by evaporation was

replaced by sterile distilled water. After cooling, the solutions were forced by air pressure through glass tubes into burettes and the proper amount of each was carefully measured into the culture flask. All glassware and stoppers were sterilized either in an autoclave or in a hot-air chamber immediately before using. In order to reduce the chances of outside contamination, the cultures were made up and inoculated in a dust-proof inoculation chamber. A number of uninoculated check cultures proved that they had been made under perfectly sterile conditions.

It would have been more convenient to make up the cultures before sterilization, but this method was not considered advisable on account of the decomposition of the salts which occurs while heating a mixed salt solution. Some of the solutions employed, especially those with a relatively large amount of KH_2PO_4, were quite unstable at the boiling point, and even at 30° C. a few of the more concentrated cultures contained a slight precipitate. The stock solutions were, therefore, sterilized separately and the culture solutions were prepared from these under aseptic conditions.

Baker's analyzed salts were used throughout. On account of the uncertainty as to the exact amount of water of crystallization in the calcium nitrate, this salt was freed from its water of crystallization by carefully fusing and dehydrating at a final temperature of 150° C. A fine grade of commercial granulated sugar was used.

The cultures were well shaken and inoculated heavily with spores from a one-week-old agar culture of *Aspergillus niger*. Enough spores were transferred in the inoculation to produce a very thin, but visible, uniform film on the surface of the culture. This heavy inoculation was found necessary to get a uniform growth. Light inoculations were apt to give "islands" of growth instead of a uniform film over the entire surface. A preliminary test to determine the possible error caused by unequal inoculation showed that the amount of inoculum may be varied considerably without affecting the yield, provided the sowing is uniform. The amount of inoculum used in these experiments could be reduced to one half or doubled without affecting the yield.

The cultures were incubated at 29° to 30° C. for seven days. It has been shown by Brenner (2) and others that *Aspergillus niger* in a good nutrient medium makes its full growth in from three to five days, and that there is a gradual loss in dry weight after that time. A seven-day growing period was chosen in this work in order to give the poorer cultures a chance to get their full development. Under the conditions of these experiments it was found that harvesting could be delayed until the seventh day without affecting the results appreciably, as was shown by an experiment to determine the growth curve in one of the best solutions used in this work (culture R1C8, series 3). In this experiment fourteen cultures were inoculated and two were harvested each day for seven consecutive days. The graph in figure 1, which is self explanatory, shows the result of this experiment. From

this graph it will be seen that although the maximum growth was reached at the end of the third day, there was only a very slight decline up to the end of the seventh day. No appreciable loss is thus sustained by allowing the growth to proceed for seven days.

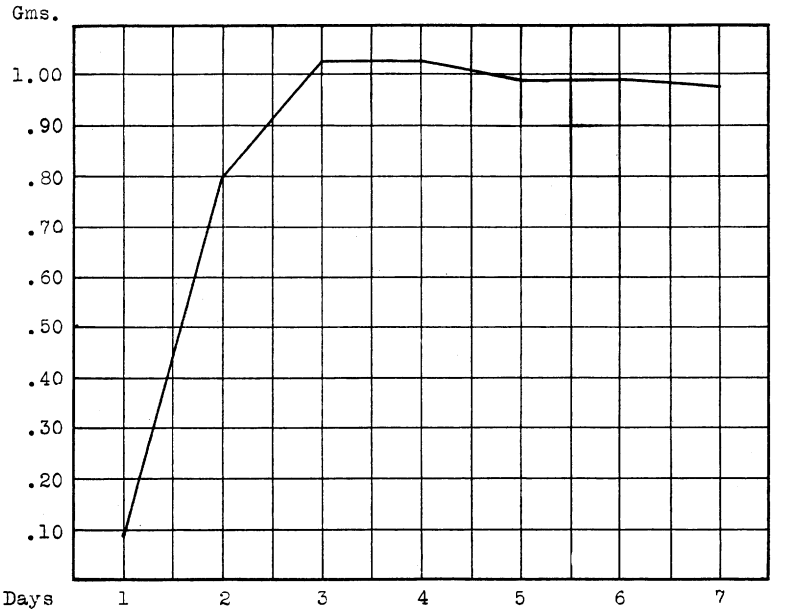

FIG. 1. Graph showing the rate of growth of *Aspergillus niger* in a three-salt medium.

At the end of the growing period the fungus was placed upon a dried and weighed filter paper, washed with water, placed with filter paper into weighing bottles, dried to constant weight at a final temperature of 104° C., and weighed to the nearest milligram.

EFFECT OF INCREASING TOTAL SALT CONCENTRATION ON THE GROWTH OF ASPERGILLUS NIGER

Table 3 gives the absolute dry weights of the fungus from the cultures in each of the five series. By reading the yields across the columns in this table, it is seen that the dry weights increase as the total salt concentration increases. This increase is shown for all corresponding cultures, regardless of the salt proportions. Figure 2, in which yields are plotted in the form of graphs, shows the relation between total concentration and yield more clearly than does table 3. In these curves the dry-weight yields of series 2 have been plotted in the descending order of their values and the yields from the other series are plotted with the same culture order as series 2. The light lines represent the yields from series 1, 2, and 3, which differ from each other, with respect to the composition of the solutions used, only in

TABLE 3. *Dry weights of fungus obtained from the cultures of series 1 to 5, and the yield ratios between cultures having the same salt proportions but varying in total concentration*

Culture No.	$Ca(NO_3)_2$ Group					$NaNO_3$ Group		
	Dry Weight of Fungus			Ratio		Dry Weight of Fungus		Ratio
	Ser. 1 (0.5 Atm.)	Ser. 2 (2.1 Atm.)	Ser. 3 (4.2 Atm.)	Ser. 2 / Ser. 1	Ser. 3 / Ser. 2	Ser. 4 (2.1 Atm.)	Ser. 5 (4.2 Atm.)	Ser. 5 / Ser. 4
R1C1...	.065	.173	.347	2.66	2.01	.098	.199	2.03
R1C2...	.114	.343	.624	3.01	1.82	.194	.382	1.97
R1C3...	.168	.474	.874	2.82	1.84	.292	.553	1.89
R1C4...	.209	.606	.956	2.90	**1.58**	.369	.709	1.92
R1C5...	.248	.762	.949	3.07	**1.25**	.457	.775	**1.70**
R1C6...	.282	.848	.983	3.01	**1.16**	.544	.754	**1.39**
R1C7...	.302	.900	.985	2.98	**1.09**	.621	.723	**1.16**
R1C8...	.328	.915	.977	2.79	**1.07**	.665	.701	**1.05**
R2C1...	.062	.174	.351	2.81	2.02	.108	.202	1.87
R2C2...	.103	.331	.632	3.21	1.91	.214	.390	1.82
R2C3...	.159	.477	.865	3.00	1.81	.282	.564	2.00
R2C4...	.199	.625	.947	3.14	**1.52**	.369	.721	1.95
R2C5...	.249	.736	.969	2.96	**1.32**	.454	.777	**1.71**
R2C6...	.281	.835	.984	2.97	**1.18**	.546	.743	**1.36**
R2C7...	.307	.900	.991	2.93	**1.10**	.610	.711	**1.17**
R3C1...	.058	.182	.355	3.14	1.95	.102	.197	1.93
R3C2...	.103	.334	.610	3.24	1.83	.193	.386	2.00
R3C3...	.151	.477	.875	3.16	1.83	.283	.560	1.98
R3C4...	.205	.605	.957	2.95	**1.58**	.373	.714	1.92
R3C5...	.243	.730	.957	3.00	**1.31**	.456	.766	**1.68**
R3C6...	.276	.824	.976	2.99	**1.18**	.551	.743	**1.35**
R4C1...	.061	.189	.341	3.10	1.80	.101	.203	2.01
R4C2...	.101	.330	.603	3.27	1.83	.193	.383	1.98
R4C3...	.159	.491	.874	3.09	1.78	.294	.565	1.92
R4C4...	.207	.600	.960	2.90	**1.60**	.369	.731	1.98
R4C5...	.231	.730	.966	3.16	**1.32**	.468	.759	**1.62**
R5C1...	.056	.180	.354	3.21	1.97	.104	.191	1.84
R5C2...	.110	.340	.634	3.09	1.86	.192	.373	1.94
R5C3...	.148	.477	.867	3.22	1.82	.274	.576	2.10
R5C4...	.203	.599	.958	2.95	**1.60**	.366	.730	1.99
R6C1...	.060	.181	.364	3.02	2.01	.106	.194	1.83
R6C2...	.119	.343	.636	2.88	1.85	.188	.378	2.01
R6C3...	.148	.479	.886	3.24	1.85	.285	.555	1.95
R7C1...	.055	.186	.352	3.38	1.89	.091	.193	2.12
R7C2...	.101	.326	.625	3.23	1.92	.204	.358	1.76
R8C1...	.059	.194	.324	3.29	1.67	.092	.192	2.09
Check	.201	.647	.733				.730	

total salt concentration. It will be noted that series 1, which has a total osmotic salt-concentration value of 0.5 atmospheres, gave relatively low yields throughout all the salt proportions. In series 2, with a total osmotic salt-concentration value of 2.1 atmospheres, the yields are very much higher, and in series 3, with a concentration double that of series 2, the yields are still higher. Likewise in series 4 and 5 (represented graphically in figure 2 by the heavy lines), markedly higher yields are shown for series 5 than for series 4 in which a much lower total salt concentration was employed.

This direct correlation between total salt concentration and yield is quite clearly brought out in figure 2 by comparing each individual culture in series 2 with its corresponding cultures in the other series. Such a compari-

son between the yields from series 1 and series 2 indicates that series 2, with a total concentration value four times that of series 1, gave yields throughout which were approximately three times as great as the corresponding yields from series 1. The exact ratios of the yields from series 2 to the corresponding ones from series 1 are given in table 3, as are also similar ratios between

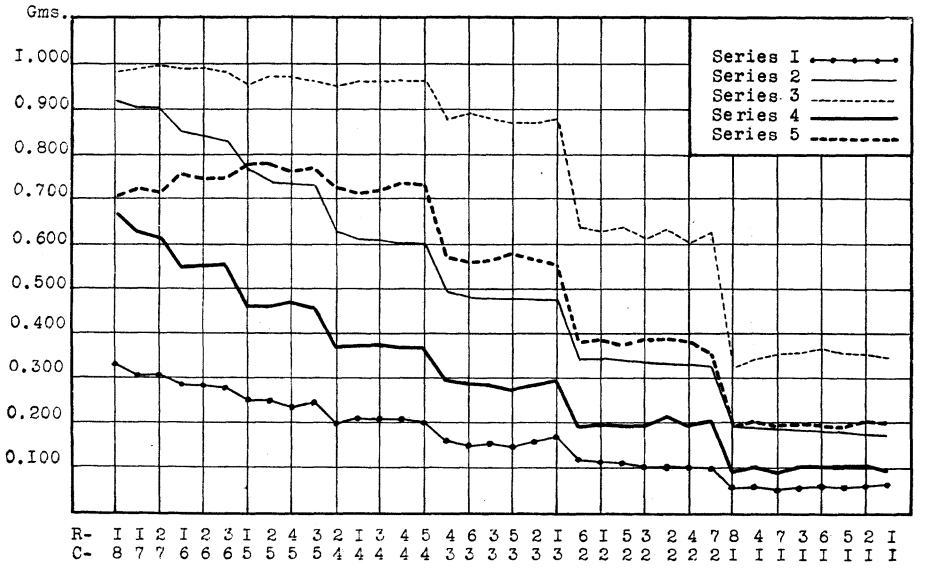

FIG. 2. Graphs showing the absolute yields from the cultures of series 1 to 5.

the yields from series 2 and 3, and between those from series 4 and 5. A relation similar to the one here pointed out between total salt concentration and yield exists throughout all the series, as is clearly shown by the graphs of figure 2, and by the columns of ratios in table 3. This relation disappears, however, in the high-yielding cultures of series 3. It was found that in these cultures the sugar supply was sufficiently exhausted to become a limiting factor for growth, thus preventing changes in the total salt concentration from showing their true effects. If these high-yielding cultures are omitted from the comparison and only the 21 cultures of series 3 which have low or medium yields are compared with the corresponding cultures of series 2, it will be found that the yields from series 3 are approximately twice as great as are the corresponding yields from series 2, while the total salt concentration in series 3 is double that in series 2. A similar comparison of the corresponding 21 cultures of series 4 and 5 shows a similar correlation between concentration and yield. The ratio of the total salt concentration between series 4 and 5 is 1 : 2, while the ratios between the yields of the corresponding cultures is also approximately 1 : 2, which indicates that by doubling the total osmotic concentration of the solutions without altering the salt proportions, the yields are also approximately

doubled, as is clearly shown by the ratios in the last column of table 3. The ratios derived from the yield values of the cultures in series 3 and 5 which do not show this relation are indicated in table 3 in bold-face type.

Effect of Varying the Salt Proportions on the Growth of Aspergillus niger

To facilitate the comparison between the yields with respect to the variations in the salt proportions, the yield values are presented in graphic form in the triangular diagrams of figures 3 and 4, in which each culture occupies a definite position according to the proportions of the salts it contains. In these triangles the individual cultures are numbered according to the row in which they occur (R1, R2, R3, etc.) and according to their position in the row (C1, C2, C3, etc.) According to this triangular diagram (6) the osmotic proportions of the salts in any culture are indicated by its position on the triangle. The partial volume-molecular concentrations corresponding to these proportions are found in table 1 or in table 2. The actual dry weight of fungus derived from each culture is given just opposite the point of the triangle representing that culture. The areas on the triangles including the high, medium, and low yields are separated by dotted lines. The area at the lower right of each triangle includes the cultures giving the highest nine yields, while the cultures giving the lowest nine yields are embraced in the area lying along the left margin of each triangle. The central region on each triangle includes the cultures giving medium yields. In series 5, however, the three cultures at the extreme lower right of the triangle fall into the medium-yield area.

Considering first the effects of changing the partial concentration of KH_2PO_4 and $MgSO_4$, it will be observed in series 1 (fig. 3) that all the C1 cultures (on the left margin of the triangle) gave approximately the same yields. All these cultures contain the same amount of calcium nitrate but differ widely in their proportions of KH_2PO_4 and $MgSO_4$. The partial concentration due to each of these latter two salts varies from 1/10 to 8/10 of the total salt concentration, but in spite of the wide differences in the partial concentrations due to these two salts, all the cultures show approximately equal yield values. This same indifference of the fungus to changes in the partial concentration of KH_2PO_4 and $MgSO_4$ is shown by all cultures in which these two salts are the only variables. The C1 cultures of any one series have very nearly equal yield values. Likewise, all the C2 cultures or C3 cultures, etc., of any particular series gave approximately equal yields. In series 1, where the total concentration is only 0.5 atmosphere, or in series 3, with a total concentration value of 4.2 atmospheres, the fungus is apparently indifferent to the wide variations in the proportions of KH_2PO_4 or of $MgSO_4$. Thus all these series point to the conclusion that within wide limits neither KH_2PO_4 nor $MgSO_4$ tends either to stimulate or to inhibit growth in *Aspergillus niger*. There is no evidence of a physiologically

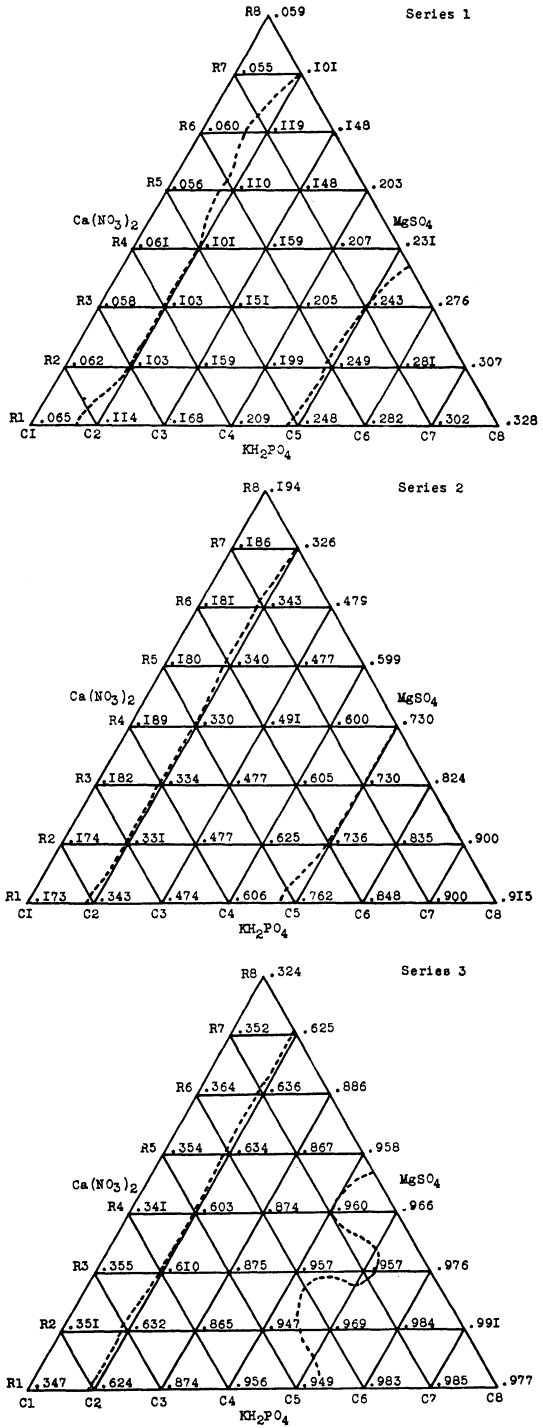

FIG. 3. Triangular diagrams showing relative yields from cultures of series 1 to 3 (group 1). Areas of low yields lie on the eft margins of the diagrams, medium yields in the central regions between dotted lines, and high yields at the lower right of each diagram.

unbalanced condition of any of the solutions in any series which can be attributed directly to variations in the proportions of these two salts.

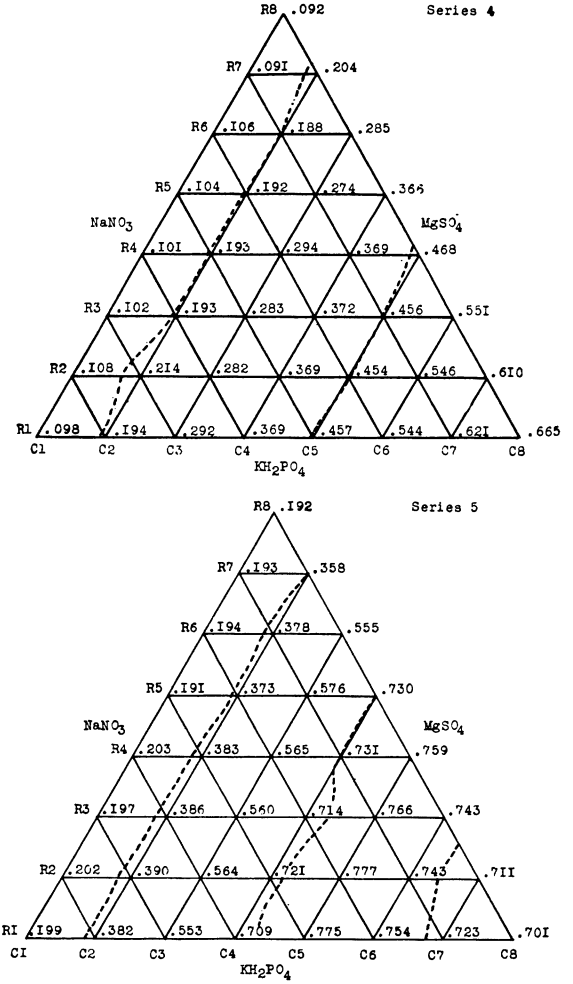

FIG. 4. Triangular diagrams showing relative yields from cultures of series 4 and 5 (group 2). Areas of low yields lie on the left margins of the diagrams, areas of medium yields in the central regions between dotted lines, and areas of high yields at the lower right of each diagram.

Steinberg (7) reported a very marked stimulative effect upon *Aspergillus niger* from high partial concentration of $MgSO_4$ and KH_2PO_4, but his results were obtained in a culture medium essentially free from iron. The addition of traces of iron gave similar stimulation, and he suggests that the increased yields in solutions with high partial concentrations of $MgSO_4$ might have been due to the heavy impurities in the $MgSO_4$ rather than to the salt

itself. The stimulative effect of high partial concentrations of KH_2PO_4 he attributes to the increased acidity in these cultures. In media containing traces of iron and zinc an increase in acidity failed to give further stimulation. From these results of Steinberg it seems probable that the iron added to the media used in the experiments herein reported was sufficient to give "maximum stimulation," and that as a consequence the $MgSO_4$ and KH_2PO_4 salts could not produce the stimulative effect which they produced in Steinberg's iron-free medium.

In direct contrast to the indifferent effect of changes in the partial concentration of KH_2PO_4 and $MgSO_4$ is the effect of changes in the partial concentration of the nitrate salts. On the triangular diagrams of series 1 to 5 (figures 3 and 4) it will be observed that for each of the series there is a uniform increase in yield values in passing from the left to the right margin of the triangle representing the series; that is, as the proportion of the nitrate salt increases, there is a corresponding increase in the yield values. In series 1, for example, in those cultures in which $Ca(NO_3)_2$ furnished only 1/10 of the total salt concentration (C1 cultures) the yields are very low, varying between 0.055 and 0.065 gram. Where the $Ca(NO_3)_2$ is increased to 2/10 of the total concentration (C2 cultures) the yields are almost doubled, the values being between 0.101 and 0.119 gram for these cultures. Likewise, throughout the entire series an increase in $Ca(NO_3)_2$ is followed by a corresponding increase in yield. Thus, culture R1C8, which has the highest nitrate content of any culture in the series, has also the largest yield (0.328 gram). This same close correlation between the partial concentration of NO_3 and yield is shown for all the series, the only apparent exception being in the high-yielding cultures of series 3 and 5 in which the exhaustion of the sugar supply limited further growth.

The relation between the partial concentrations of NO_3 and yields is brought out very clearly in figure 5, which shows the dry weight yields plotted against NO_3 content of the cultures. In these graphs the abscissas represent grams of NO_3 per liter of culture medium, and the ordinates represent grams dry weight of fungus per culture. Each dry weight represents the average of all cultures of a single series containing equal quantities of NO_3.

It will be observed that the curves representing series 1, 2, and 3 (Ca group) are very nearly coincident, indicating that all cultures within this group which contain equal quantities of NO_3 have approximately the same yield values regardless of the total salt concentration or of the amounts of KH_2PO_4 and $MgSO_4$ present. This fact is brought out very clearly if a direct comparison is made between the yield values from cultures with the same NO_3 content but having different total concentrations. Thus, culture R1C8 of series 1, all the C2 cultures of series 2, and all the C1 cultures of series 3 contain approximately the same amount of NO_3 per liter, but differ widely in their total salt concentrations. Culture R1C8 of series 1

gave a yield of 0.328 gram. The average yield from the C2 cultures of series 2 is 0.333 gram, and from the C1 cultures of series 3 it is 0.348 gram. This agreement is extremely close when it is considered that the partial concentrations due to either KH_2PO_4 or $MgSO_4$ in these cultures vary from less than 0.1 atmosphere in series 1 to more than 3.0 atmospheres in series 3. A similar equality between dry-weight yields exists for all the cultures containing equal quantities of NO_3 in the group comprising series 1, 2, and 3. This same relation exists also in the group comprising series 4 and 5. The direction of the graph of each series with a given total salt concentration indicates an approximately linear relation between the dry-weight yields of the fungus and the proportions of NO_3 in the media.

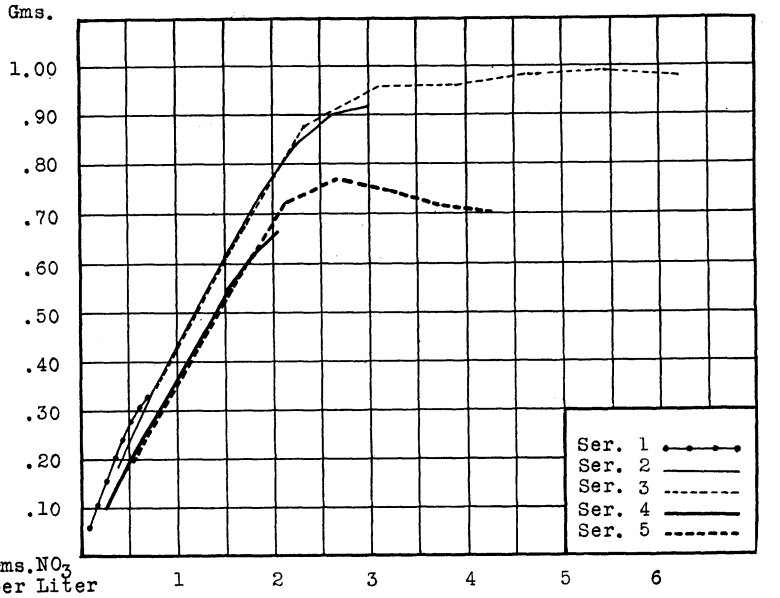

FIG. 5. Graph showing relation between dry-weight yields of *Aspergillus niger* and the NO_3 content of the nutrient solution.

It will be observed that the graphs of series 3 and 5 (fig. 5) break abruptly at the point where high yields are indicated. This sudden break itself, according to Blackman (1), indicates that a limiting factor for growth had entered at this point, and, as already pointed out, this is attributed to the exhaustion of the sugar content in these high-yielding cultures.

That growth in the two series in question was limited by the amount of sugar available in the cultures is clearly brought out in a series of eight duplicate cultures in which the sugar content was made to vary from a calculated osmotic concentration value of 1.0 atmosphere to one of 8.0 atmospheres, by increments of one atmosphere, the total salt concentration and the salt proportions remaining constant in all the cultures. The

solution of culture R1C8 in series 3 was used in this test. This solution has a total salt-concentration value of 4.2 atmospheres, and the three salts KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$ are present in the proportions of 0.0710 m., 0.0063 m., and 0.0108 m., respectively.

The results of this test are shown in the graph of figure 6 in which the ordinates represent dry weights in grams and the abscissas represent concentrations of sugar in atmospheres. This graph brings out the fact that as the sugar concentration is increased (and therefore the amount of sugar per culture, since the amount of solution was the same in all the cultures, namely 50 cc.), the dry-weight yields were proportionately higher, thus showing a linear relation between the amounts of sugar employed and the yield values.

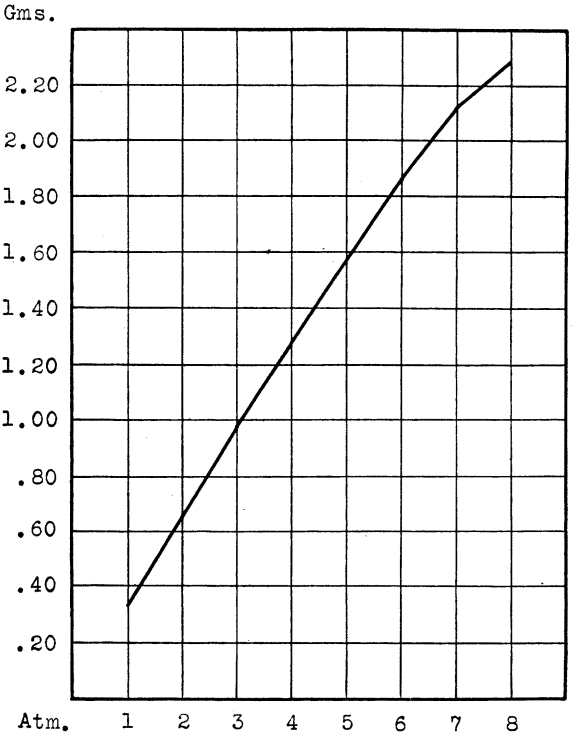

FIG. 6. Graph showing relation between dry-weight yields of *Aspergillus niger* and the sugar content of the nutrient solutions.

It is to be observed that the culture with a sugar content of 3 atmospheres gave an average yield of 0.974 gram, which is nearly the same as the yield from culture R1C8 of series 3 having the same composition, the yield obtained from this latter culture being 0.977 gram. However, the yields continued to increase proportionately with increase in sugar concentration up to a concentration of 8 atmospheres, at which concentration the dry

weight yield was 2.283 grams. It is thus clear that with 50 cc. of solution having a sugar concentration of 3 atmospheres, the maximum yield which can be produced is between 0.9 gram and 1.0 gram, the point at which the curve of series 3 (fig. 5) breaks abruptly.

Effect upon Aspergillus niger of Substituting $NaNO_3$ for $Ca(NO_3)_2$ in the 3-salt Solutions

A microscopic examination of the fungus from the cultures containing $Ca(NO_3)_2$ showed a slight precipitate of calcium oxalate crystals upon the hyphae. Not all of this deposit was removed by the washing process to which the fungus was subjected before drying. The amount of calcium oxalate crystals which remained after washing was not determined, so that it can not be stated definitely to what extent the dry weights were affected by it, but it is certain that increase in weight due to adhering crystals which remained after the washing process was small. However, since there is a possibility of the yield values of series 1, 2, and 3 being slightly higher than they should be, and since the formulae of media used for the growth of fungi do not ordinarily contain calcium nitrate, series 2 and 3 were repeated, substituting sodium nitrate in equivalent osmotic partial concentrations for the calcium nitrate in the various cultures of the corresponding series. These two series have already been referred to as series 4 and 5 of the $NaNO_3$ group, and the volume-molecular salt proportions of the solutions used are given in table 2. The yields from these two series are given in table 3 and plotted in the graphs of figure 5 in connection with those from the series of the $Ca(NO_3)_2$ group. The yields are also shown on the triangular diagrams of figure 4 which have already been considered.

A comparison of the graphs (fig. 2) shows at once that the yields from series 4 and 5 are uniformly much lower than are the corresponding yields from series 2 and 3 respectively. Thus, substituting in any culture $NaNO_3$ for $Ca(NO_3)_2$ in equivalent osmotic concentrations had the effect of reducing the yield very considerably. This great reduction in yield is largely accounted for by the fact that the nitrate content of the cultures in series 4 and 5 is only 68.3 percent of the NO_3 content in the corresponding cultures of series 2 and 3 respectively, this difference in the NO_3 content of the cultures being due, of course, to the difference in the composition and osmotic value of the nitrate salts employed. That this large difference in yields, however, is not entirely due to the difference in the NO_3 content of the cultures is clearly brought out by the graphs of figure 5. It will be observed that the graphs representing series 1, 2, and 3 lie throughout above the graphs of series 4 and 5, thus indicating that with equivalent amounts of NO_3 per culture (grams per liter of nutrient solution) the cultures containing $Ca(NO_3)_2$ gave uniformly higher yields than did the cultures containing $NaNO_3$.

The exact cause of these observed differences in yield is not clear. They

are doubtless in part due to the deposit of calcium oxalate crystals on the hyphae in the $Ca(NO_3)_2$ series, but the differences are apparently too great to be entirely attributed to this cause. The reduced yield in the $NaNO_3$ group may in part be the result of a toxic influence of the Na, as seems to be indicated by the reduction in yield in the cultures containing the largest amounts of $NaNO_3$. This reduction in yield is indicated by the downward trend of the upper end of the curve representing series 5 (fig. 5). There is also a possibility that the absence of Ca has somewhat depressed the yields in the $NaNO_3$ group. Although it has been demonstrated that calcium is not essential to the growth of fungi, it may still be beneficial as an antagonizing influence in some such manner as has been suggested by Osterhout (5), who states that

> The classical researches of Pasteur and Raulin and the later work of other investigators have shown that calcium is not needed for the nutrition of fungi, and it is, therefore, omitted from culture solutions for these plants. This answers very well as long as the solutions are sufficiently dilute. I find, however, that when the concentration of the solution is increased it becomes toxic, and the addition of calcium then produces a remarkable improvement in growth. Calcium, therefore, has a protective value for fungi just as for other plants, though not needed for nutrition.

SUMMARY

Aspergillus niger was grown on three-salt solutions of total concentrations equivalent to 0.5, 2.1, and 4.2 atmospheres respectively. For each total concentration 36 solutions were made, representing all the possible combinations obtained by varying the partial concentrations of each of the salts by increments of 1/10 of the total concentration.

A number of solutions in which the salt proportions and total salt concentration remained the same, but with sugar concentrations varying from 1 to 8 atmospheres by increments of one atmosphere, were also tested.

1. In solutions all with the same salt proportions, an increase in total concentration gave a corresponding increase in yield.

2. The partial concentrations of KH_2PO_4 and $MgSO_4$ were varied within wide limits without in any way affecting the yields.

3. Yield in dry weight of fungus is approximately proportional to the amount of NO_3 present in the culture, whether this is produced by increasing total concentration and leaving salt proportions unchanged, or by changing salt proportions and leaving total concentration the same.

4. With a sugar solution having an osmotic concentration value of three atmospheres, the limit of growth in the $Ca(NO_3)_2$ cultures was between 0.9 gram and 1.0 gram, regardless of the amount of salts present.

5. In cultures with constant salt proportions and total salt concentrations but with varying sugar concentrations, the dry weights of fungus were very nearly proportional to the sugar concentrations of the cultures.

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